

Amendments to the Specification:

Please amend the following paragraphs as indicated:

On page 12, please replace paragraph [0020]:

[0028] Figure 7 is a bar graph showing competitive inhibition of high affinity peptides to rhodopsin by heterotrimeric heterotrimeric Gt.

On page 21, please replace paragraph [0053]:

[0053] To produce very high affinity peptide GPCR blockers, the tertiary structure of a wild-type G α carboxyl terminal peptide or any other GPCR binding peptide in its receptor-bound conformation may be studied, for example, using trNOESY (NMR). Dratz et al., Nature, 363:276-280, 1993. Structural data derived from these types of studies of G protein regions are combined with analysis of activity of substituted peptide analogs to define the minimal structural requirements for interaction of peptides with GPCR. The following experimental systems are examples of systems which can be used to define receptor-G protein interactions: (i) rhodopsin-transducin (G α t) in retinal rod cells, (ii) β -adrenergic receptor-G α s in C6 glioma cells, (iii) adenosine A1 receptor-G α 1 in Chinese hamster ovary cells, (iv) GABA $_B$ receptors-G α 1 in rat hippocampal CA1 pyramidal neurons, (v) muscarinic M2 receptor-G α 1 in human embryonic kidney cells, and the like. Any GPCR or group of GPCR which is convenient or desired can be used to define the interaction requirements, and skilled workers are aware of many methods to understand structure-activity relationships in receptor binding of this kind. Any of these methods are contemplated for use in these methods and may substitute for the particular methods of the exemplified embodiment.

On page 32, please replace paragraph [0064]:

[0064] One preferred ELISA, where signal strength is better correlated with affinity, involves fusing the sequences of interest from a population of clones in frame with the gene encoding a protein, for example maltose binding protein (MBP). Once the sequences have been transferred into the monomeric fusion protein, they can be overexpressed in *E. coli* and used as either crude lysates or purified fusion proteins for assay by an ELISA which detects the protein bound to receptor or any convenient assay. Those samples with an ~~absorbance~~ absorbence of at least two standard deviations above background may be considered to contain high affinity binding peptides. Any desired cut-off point may be used, however, depending on the assay parameters and the needs of the operator. The purified fusion proteins can be further tested by measuring their ability to compete for the site of binding on the receptor using native peptide, a LacI-peptide fusion protein, or heterotrimeric G protein. Use of competitive ELISA allows one to calculate IC₅₀ values for the binding of individual fusion protein to the immobilized receptor.

On page 35, please replace paragraph [0069]:

[0069] Any method known in the art for selecting and synthesizing small molecule libraries for screening is contemplated for use in this invention. Small molecules to be screened are advantageously collected in the form of a combinatorial library. For example, libraries of drug-like small molecules, such as β -turn mimetic libraries and the like, may be purchased from for example ChemDiv (~~<http://www.chemdiv.com>~~), Pharmacopia (~~<http://www.pcop.com>~~ or Combichem (~~<http://www.combichemlab.com>~~) or synthesized and are described in Tietze and Lieb, *Curr. Opin. Chem. Biol.* 2:363-371, 1998; Carrell

et al., Chem Biol. 2:171-183, 1995; United States Patent No. 5,880,972, United States Patent No. 6,087,186 and United States Patent 6,184,223. Any of these libraries known in the art are suitable for screening, as are random libraries or individual compounds. In general, hydrophilic compounds are preferred because they are more easily soluble, more easily synthesized, and more easily compounded. Compounds having an average molecular weight of about 500 often are most useful, however, compounds outside this range, or even far outside this range also may be used. Generally, compounds having c logP scores of about 5.0 are preferred, however the methods are useful with all types of compounds. Simple filters like Lipinski's "rule of five" have predictive value and may be used to improve the quality of leads discovered by this inventive strategy by using only those small molecules which are bioavailable. See Lipinski et al., Adv. Drug Delivery Rev. 23:3-25, 1997.

On pages 41-42, please replace paragraph [0083]:

[0083] Murine leukemia virus (MLV) derived retroviral vectors are commonly used vehicles for stable delivery of therapeutic genes into endothelial cells. For the retrovirus studies in vivo, high affinity peptides subcloned into a replication-defective murine Moloney retrovirus vector which is Tet-inducible and co-expresses GFP driven by an internal ribosomal entry site (IRES) from encephalomyocarditis virus (pTet-GFP). These constructs may then be transiently transfected into producer line to generate cell-free titers of ~~10^6-10^7 i.u./ml~~ 10^6-10^9 i.u./ml. If needed, a pantropic retroviral expression system (GP-293; Clontech) which utilizes VSV-G, an envelope glycoprotein from the vesicular stomatitis virus, may be utilized to overcome low transfection efficiencies. By using this innovative cell-based gene transfer method one can obtain stable, long-term, and

localized gene expression of the high affinity C-terminal peptides.

On page 50, please replace paragraph [0100]:

[0100] Construction of a biased peptide library has been described previously. Martin et al., J. Biol. Chem. 271:361-366, 1996; Schatz et al., Meth. Enzymol. 267:171-191, 1996. The vector used for library construction was pJS142 (see Figure 2). This vector had a linker sequence between the LacI and the biased undecamer undecamer peptide coding sequence, as well as restriction sites for cloning the library oligonucleotide. The oligonucleotide synthesized to encode the mutagenesis library was synthesized with 70% of the correct base and 10% of each of the other bases at each position. This mutagenesis rate leads to a biased library such that there is approximately a 50% chance that any of the 11 codons will be the appropriate amino acid and approximately a 50% chance that it will be another amino acid. In addition, a linker of four random NNK (where N denotes A, C, G or T and K denotes G or T) codons were synthesized at the 5' end of the sequence to make a total of 15 randomized codons. Using this method, a library with greater than 10^9 independent clones per microgram of vector used in the ligation was constructed based on the carboxyl terminal sequence of Gxt (IKENLKDCGLF; SEQ ID NO:139). The nucleic acid used for creating this library was 5'-GAGGTGGTNNKNKKNNKNNKtcaaggagaaacctgaaggactgcccctcttcTAACATAAGTAAAGC-3', wherein N= A/C/G/T and K= G/T; SEQ ID NO:140).

On pages 51-52, replace paragraph [0111]:

[0102] Sf9 cells (2×10^8 cells) were cultured with 200 ml of Grace's insect cell culture medium (Life Technologies, Inc., Grand Island, NY) containing 0.1% Pluronic F-68 (Life Technologies, Inc., Grand Island, NY), 10% fetal calf serum, and

20 µg/ml gentamicin in a 1-liter spinner flask at 27°C for 25 hours. Sf9 cells were infected with the ThR/pBluebac recombinant virus at a multiplicity of infection of 3-5, and cultured at 27°C for 4 days. The cells were harvested, washed with phosphate buffered saline, and then resuspended in 10 mM Tris-HCl, pH 7.4. Cells were then ~~homogenized~~ homogenized with a hand-held ~~homogenizer~~ homogenizer set at low speed for 20 seconds. The broken cells were then sedimented at 17,000 x g for 15 minutes. The supernatant was discarded, and the pellet resuspended in a buffer consisting of 50 mM Tris-HCl, pH 7.4 and 10% glycerol. Concentration of receptor in the membrane preparation ranged from 1-10,000 pM/mg. For screening, a final concentration of 200 µg/ml was used. The thrombin receptors were tested for their ability to bind to the native Gq-C terminal peptide using a MBP-Gq fusion protein. (Figure 7).

On page 58, replace paragraph [0112]:

[0112] To transfect, aliquots (40 µL) of thawed ARI814 cells were placed into each of three chilled microcentrifuge tubes. A peptide display library based on the undecamer carboxyl terminal peptide of Gα_i (SEQ ID NO:126) was prepared according to Example 1. Two microliters of library plasmid were added to the tubes and mixed. For the first round of "panning," 200 ~~µL~~ µL of the plasmid library was added. For subsequent rounds, three sets of transfections were performed (adherent plasmids from wells containing receptor (+); adherent plasmids from wells containing no receptor (-); and the PRE sample which was not incubated). See below. In each round of panning, less library was used (round 2:100 ~~µL~~ µL; round 3:50 ~~µL~~ µL; round 4:10 ~~µL~~ µL). After the panning was completed, the DNA for the LacI fusion protein is eluted. This DNA (50 ~~µL~~ µL) is used to transfect E. Coli cells by electroporation, using cold, sterile 0.1 cm

electrode gap cuvettes. The cuvettes were pulsed one time using a BioRad E. coli Pulsar set to 1.8 kV, 25 μ F capacity, time constant 4-5 mseconds, with the Pulser Controller unit at 200 m Ω . Immediately, 1 mL of SOC was added and the mixture transferred to a labeled 17 x 100 mm polystyrene tube. The tube was shaken for one hour at 37°C. Aliquots were taken from each set to plate 100 μ L undiluted to 10⁻⁶ dilution samples on LB-Amp plates. Counts of the PRE plates indicated library diversity, while comparison of the (+) and (-) plates indicated whether specific clones were being enriched by the panning procedure.

On page 59, please replace paragraph [0113]:

[0113] The remaining ~900 μ L in the + receptor tube was added to a 1L flask containing 200 mL LB-AMP media, prewarmed to 37°C, and grown at 37°C, shaking until OD₆₀₀ = 0.5. The tube of cells then were placed in an ice water bath for at least 10 minutes, and kept chilled at or below 4°C during the subsequent washing steps. The cells were sedimented at 5000 xg for six minutes, resuspended in 100 mL WTEK buffer, sedimented at 5000 xg for six minutes, resuspended in 50 mL TEK buffer, resedimented at 5000 xg for six minutes and ~~resuspended~~ resuspended in 4 mL HEK buffer. The cells were divided into the cryovials and stored at -70°C. One tube was used for the next round of panning and the other saved as a backup.

On page 59, please replace paragraph [0114]:

[0114] The panning process is illustrated in Figure 1. For screening of the library by "panning," rhodopsin receptors prepared according to Example 5 were immobilized directly on Immulon 4 (Dynatech) microtiter wells (0.1-1 μ g of protein per well) in cold 35 mM HEPES, pH 7.5, containing 0.1 mM EDTA, 50 mM KCl and 1mM dithiothrietol (HEK/DTT). After shaking for one hour

at 4°C, unbound membrane fragments were washed away with HEK/DTT. The wells were blocked with 100 ~~µl~~ µL 2% BSA in HEKL (35 ~~Mm~~ mM HEPES; 0.1 mM EDTA; 50 mM KCl; 0.2 M α-lactose; pH 7.5, with 1 mM DTT). For rounds 1 and 2, BSA was used for blocking; in later rounds 1% nonfat dry milk was used. For the first round of panning, about 24 wells of a 96-well plate were used. In subsequent rounds, 8 wells with receptor and 8 wells without receptor were prepared.

On pages 68-69, please replace paragraph [0121]:

[0121] In the last round of panning, several clones were selected from the (+) receptor plates and grown up overnight in LB-Amp media. Three hundred microliters of the overnight culture was diluted in 3 mL in LB-Amp media for "ELISA lysate culture." Another 30 μ L was added to an equal volume of 50% glycerol was stored in labeled microcentrifuge tubes at -70°C. The remaining 4.5 mL was used to make DNA using a standard miniprep protocol (Qiagen Spinprep™ kits) and sequenced using a 19 base pair reverse primer which is homologous to the vector at a site 56 basepairs downstream from the TAA stop ~~codon~~ codon that terminates the random region of the library (GAAAATCTTCTCTCATCCG; SEQ ID NO:306). The DNA was stored at -20°C. The ELISA lysate culture was allowed to shake for one hour at 37°C. Expression was induced by adding 33 μ L 20% arabinose (0.2% final concentration) with shaking at 37°C for 2-3 hours. The culture then was subjected to sedimentation at 4000 xg for five minutes, the pellet resuspended in 3 mL cold WTEK buffer, resedimented at 4000 xg for five minutes and the pellet resuspended in 1 mL cold TEK buffer. After transfer to 1.5 mL microcentrifuge tubes, the pellet was sedimented at 13,000 xg for two minutes and the supernatant aspirated. The cell pellet was resuspended in 1 mL lysis buffer (42 mL HE, 5 mL 50% glycerol, 3 mL 10 mg/mL BSA in

HE, 750 μ L 10 mg/mL lysozyme in HE and 62.5 μ L 0.2 M PMSF) and incubated on ice for one hour. One hundred ten microliters 2M KCl was added to the lysis mixture and inverted to mix, then sedimented at 13,000 xg for 15 minutes at 4°C. The clear crude lysate (about 0.9 mL supernatant) was transferred to a new tube and stored at -70°C.

On pages 69-70, please replace paragraph [0122]:

[0122] The binding properties of the peptide encoded by individual clones were assayed as follows. Purified PAR1 receptor prepared from Sf9 insect cells (1-10,000 pg/mL in 50mM Tris ~~HCl~~, HCl pH 7.4, 10% glycerol) was reconstituted in lipid vesicles according to Example 6. A serial dilution of the membranes containing receptor ranging from 0.2 to 20,000 μ g/mL (+/- receptor) was added to wells on a microtiter plate and shaken gently for one hour at 4°C. After washing, a 1:1 to 1:10,000 serial dilution of a LacI-Gq lysate prepared from the LacI-Gq clone according to the methods described in Example 12 was added to the wells, the plate was shaken gently for one hour at 4°C, and washed. Anti-LacI antibodies (Stratagene) were added (1:1000) and the plate shaken gently for one hour at 4°C. After washing, HRP-conjugated goat anti-rabbit antibodies (Kierkegaard and Perry Laboratories) were added (1:2500) and the plate shaken gently for one hour at 4°C. The plate was washed, color was developed using horseradish peroxidase, and then read in an ELISA reader at OD₄₅₀. The general methodology for the ELISA is illustrated in Figure 3. The results, see Figure 4, show that the LacI-Gq fusion protein binds thrombin receptor in a concentration dependent manner. The ability of the LacI-Gq fusion protein to bind the empty vesicles was significantly less than vesicles reconstituted with thrombin receptor.

On pages 75-76, please replace paragraph [0130]:

[0130] For the assay, in the dark, 1 μ g/well of ROS membranes (rhodopsin) as described in Example 5 was directly immobilized on microtiter wells in cold HEK/DTT for one hour at 4°C. The wells were rinsed, blocked with 1% BSA in HEK/DTT for one hour at 4°C and rinsed again. Bound rhodopsin was activated by exposure to light for 5 minutes on ice before addition of the MBP fusion proteins (crude bacterial lysates were diluted 1:50 in HEK with 1 μ M dithiothreitol; purified proteins were used at 0.2-120 nM). The MBP-G α t340-350K341R (pELM17) fusion protein and MBP with linker sequence only (pELM6) were present in control wells at 50nM final concentration. After 30 minutes, wells were washed and rabbit anti-MBP antibody (New England Biolabs) was added. The anti-MBP antibody was used at a 1:1000 dilution for crude lysates and a 1:3000 dilution for purified proteins. After 30 minutes, wells were rewashed and goat anti-rabbit antibody conjugated to horseradish peroxidase (1:7500 dilution for crude lysates; 1:10,000 dilution for purified proteins; Kierkagaard & Perry Laboratories) was added. After 30 minutes, the plate was washed four times with PBS containing 0.05% TweenTM20. Horseradish peroxidase substrate (100 ~~μl~~ μL) was added and color was allowed to develop for about 20 minutes. The reaction was stopped by addition of 100 ~~μl~~ μL 2N sulfuric acid. The results are presented in Figure 6. Values indicate ~~absorbance~~ absorbance at OD₄₅₀. The controls for the assay was pELM 17, which encodes the MBP fusion protein G α t340-350K341R. pELM6, which expresses MBP protein fused to a linker sequence only, served as the negative control. "No lysate" control wells were included to reflect any intrinsic, non-specific binding within the assay. See Figure 6.

On page 77, please replace paragraph [0132]:

[0132] Binding of MBP fusion proteins containing the high affinity peptide from the library (sequences from clones 8, 9, 10, 18, 23, 24, as well as pELM17 which encodes the wild-type peptide sequence, and pELM6 which contains on peptide) were assessed for their ability to bind rhodopsin (0.5 μ g rhodopsin/well) in the presence or absence of heterotrimeric Gt. Lysate (50 μ l) from each clone was added and incubated in the light. After 45 minutes, 1 μ M heterotrimeric Gt was added and the solution incubated for 30 minutes. Anti-MBP antibody was added, followed by goat anti-rabbit alkaline phosphatase conjugated antibody and substrate. The color was allowed to develop. ~~Absorbance~~ Absorbance data are presented in Figure 7.

On page 78, please replace paragraph [0135]:

[0135] Microtiter wells were coated with purified, reconstituted PAR1 in the presence of 100 nmoles thrombin receptor activating peptide, as described above in Example 6. Purified maltose binding protein-G α q (MBP-G α q) was added at the concentrations indicated in Figure 10 and incubated one hour on a shaker at 4°C. The wells were rinsed and then probed with a rabbit anti-maltose binding protein antibody, followed by alkaline phosphatase conjugated secondary antibodies, as described above. Substrate was added and the color was allowed to develop about 20 minutes. ~~Absorbance~~ Absorbance at 405 nm was measured and dose-response curves were calculated using GraphPad Prism (version 2.0). See results in Figure 10. The calculated IC₅₀ of G α q binding to activated PAR1 was 214 nM.

On page 79, please replace paragraph [0136]:

[0136] cDNA encoding the last 11 amino acids of G α subunits was synthesized (Great American Gene Company) with newly engineered

5'- and 3'- ends. The 5'- end contained a BamHI restriction enzyme site followed by the human ribosome-binding consensus sequence (5'- GCCGCCACC-3'; SEQ ID NO:314), a methionine codon (ATG) for translation initiation, and a glycine codon (GGA) to protect the ribosome binding site during translation and the nascent peptide against proteolytic degradation. A HindIII restriction enzyme site was synthesized at the 3' end immediately following the translational stop codon (TGA). Thus, the full-length 56 bp oligonucleotide for the G $\alpha_{1/2}$ carboxyl terminal sequence was 5'-

gatccgccccaccatgggaatcaagaacaacctgaaggactcggcctcttctgaa -3' (SEQ ID NO:315) and the complimentary strand was 5'-

~~agctttcagaagaggccgcagtccttcagggtgttcttgattcccatggtgg-cggcg-3' 5'-~~
agctttcagaagaggccgcagtccttcagggtgttcttgattcccatggtggcggcg-3' (SEQ

ID NO:316). See Figure 11. As a control, oligonucleotides encoding the G $\alpha_{1/2}$ carboxyl terminus in random order (G α iR) with newly engineered 5'- and 3'- ends also were synthesized. The DNA was diluted in sterile ddH₂O to form a stock concentration at 100 μ M. Complimentary DNA was annealed in 1X NEBuffer 3 (50 mM Tris-HCl, 10 mM MgCl₂, 100 mM NaCl, 1 mM DTT; New England Biolabs) at 85°C for 10 min then allowed to cool slowly to room temperature. The DNA then was subjected to 4% agarose gel electrophoresis and the annealed band was excised. DNA was purified from the band using a kit, according to the manufacture's protocol (GeneClean II Kit, Biol01). After digestion with each restriction enzyme, the pcDNA 3.1(-) plasmid vector was subjected to 0.8% agarose gel electrophoresis, the appropriate band cut out, and the DNA purified as above (GeneClean II Kit, Biol01). The annealed/cleaned cDNA was ligated for 1 hour at room temperature into the cut/cleaned pcDNA 3.1 plasmid vector (Invitrogen) previously cut with BamHI and HindIII. For the ligation reaction, several different ratios of

insert to vector cDNA (ranging from 25 μ M:25 pM to 250 pM:25 pM annealed cDNA) were plated. Following the ligation reaction, the samples were heated to 65°C for 5 min to deactivate the T4 DNA ligase. The ligation mixture (1 ~~ml~~ μ L) was electroporated into 50 ~~ml~~ μ L competent cells as described in Example 7 and the cells immediately placed into 1 ~~ml~~ μ L of SOC (Gibco). After 1 hour shaking at 37°C, 100 ~~ml~~ μ L of the electroporated cells containing the minigene plasmid DNA was spread on LB/Amp plates and incubated at 37°C for 12-16 hours. To verify that insert was present, colonies were grown overnight in LB/Amp and their plasmid DNA purified (Qiagen SpinKit). The plasmid DNA was digested with NcoI (New England Biolabs, Inc.) for 1 hour at 37°C and subjected to 1.5% (3:1) agarose gel electrophoresis. Vector alone produced 3 bands. When the 56 bp annealed oligonucleotide insert is present, there is a new NcoI site resulting in a shift in the band pattern such that the digest pattern goes from three bands (3345 bp, 1352 bp, 735 bp) to four bands (3345 bp, 1011 bp, 735 bp, 380 bp). See Figure 12. DNA with the correct electrophoresis pattern was sequenced to confirm the appropriate sequence. This method may be used to insert any high affinity peptide to create a minigene constant.

On page 82, please replace paragraph [0139]:

[0139] To verify that the peptide was being produced in the transfected cells, the cells were ~~lyzed~~ lysed and homogenized 48 hours post transfection according to known methods. Cytosolic extracts were analyzed by gradient reversed phase HPLC as follows: 100 μ L of cytosolic fraction extract was loaded onto a C4 column (Vydac) equilibrated with 0.1% TFA in ddH₂O. The peptide was eluted using 0.1% TFA in an acetonitrile gradient which increased from 0-60% over 45 minutes. Peaks were collected, lyophilized, and analyzed using ion mass spray

analysis (University of Illinois-Urbana Champagne). Mass spectrometry analysis for peak 1 from G α _{1/2} peptide vector (pcDNA-G α i) transfected cells, and from cells transfected with pcDNA-G α iR indicated that a 1450 Dalton peptide (the expected molecular weight for both 13 amino acid peptide sequences) was present in each cytosolic extract. The minigene-encoded peptides were the major peptides found in the cytosol, strongly indicating that the vectors produced the appropriate peptide sequences in large amounts.

On pages 85-86, please replace paragraph [0144]:

[0144] pcDNA, pcDNA-GiR, pcDNA-Gi, pcDNA-Gq, or pcDNA-Gs minigene constructs were transfected into HMEC and used to assay inositol phosphate (IP) accumulation 48 hours later. After 24 hours, cells were reseeded onto 24-well plates and labeled with [³H]-myoinositol (2 μ Ci/ml). After 48 hours, cells were rinsed, and incubated with or without thrombin (10 nM) for 10 minutes. Total IP accumulation was assayed as described above using Dowex™ columns to separate [³H] IP. The relative amount of [³H] IP generated was calculated as follows: (³H] IP (cpm)/[³H] IP (cpm) + [³H] inositol (cpm)). Each value was normalized by the basal value (no thrombin stimulation) obtained in pcDNA transfected cells. See Figure 16. The results presented are the normalized mean \pm SEM of at least three independent experiments performed in triplicate. The ** symbol indicated $p < 0.005$.

On page 92, please replace paragraph [0154]:

[0154] Different measures of G-protein signaling final actions were assayed in human microvascular endothelial cells (HMEC) which natively express the thrombin receptor, PAR1. The cells were seeded onto 6-well plates at 1×10^5 cells/well and transiently transfected after 24 hours with minigene constructs

containing G α carboxyl terminal peptides (pcDNA, pcDNA-G α i, or pcDNA-G α iR; 1 μ g per well) using Effectene (Qiagen) according to the manufacturer's protocol. After 24 hours, the cells were labeled with 3 μ Ci/ml [3 H]-adenine for 30 minutes at 37°C. After another 24 hours, the cells were washed with serum-free medium containing 1 mM isobutyl-methyl ~~xanthine~~ xanthine. To stimulate cAMP accumulation, cells were treated with 1 μ M isoproterenol for 30 minutes at 37°C. To see the inhibitory effects of thrombin on cAMP accumulation, cells were pretreated with thrombin (50 nM) for 15 minutes prior to addition of isoproterenol. The reactions were terminated by aspiration of media followed by addition of ice-cold 5% trichloroacetic acid.